Direct Solvent Extraction of Acid/Neutral Drugs from Biological Fluids

1 Introduction

This procedure detects common acidic and neutral drugs in biological fluids. It is derived from A Gas Chromatographic Screening Procedure for Acidic and Neutral Drugs in Blood published in the *Journal of Analytical Toxicology* by Foerster, et al (1979). While the published procedure using direct solvent extraction of fluids is followed intact to prepare a crude acid/neutral drug isolate, the drug detection method performed on this extract has been modified from gas chromatography-flame ionization detection (GC/FID) to gas chromatography-mass spectrometry (GC/MS). This latter method of detection lends a greater degree of drug specificity and sensitivity than that possessed by the original published procedure.

2 Scope

This procedure allows for screening and confirmation of a wide variety of acidic and neutral drugs in biological fluids. Dilutions[1] of food and beverage samples can also be analyzed to screen for acidic and neutral drugs. This document applies to Chemistry Unit case working personnel who perform toxicology analyses.

3 Principle

Biological specimens are screened for acidic and neutral drugs. Specimens are mixed with an internal standard (methylphenylhydantoin), adjusted to an acidic pH, and extracted with an ether:toluene mixed solvent. Following centrifugation, the organic solvent is taken to dryness and the residue is partitioned between ethanol and hexane. The ethanol layer is taken to dryness and the extract is reconstituted in a chloroform/methanol mixture prior to analysis by GC/MS.

4 Specimens

This procedure uses a biological fluid such as: blood, serum, plasma, urine, vitreous humor, or a prepared tissue homogenate. When available, 0.5 mL of blood or other fluids are used. This procedure may also be used to screen food and beverage samples for acidic and neutral drugs, provided that appropriate controls are simultaneously analyzed. A 0.5 g sample of a food or beverage homogenate or dilution is suggested for analysis.

[1] Typically, tenfold dilutions of food and beverage samples are appropriate, but case history and the complexity of the matrix may suggest a different dilution.

5 Equipment/Materials/Reagents

a. 16 x 125 mm screw-top tubes with Teflon insert caps

b. 16 x 100 mm culture tubes with polypropylene snap-tops

c. 10 x 75 mm and 12 x 75 mm culture tubes with polypropylene snap-tops

d. N- Hexane (95% or equivalent)

e. Potassium phosphate, monobasic (ACS grade or equivalent, KH_2PO_4)

f. Potassium Phosphate Buffer Monobasic (5% w:v, pH 4.5):
To a 100-mL volumetric flask, add 80 mL deionized water. Add 5 g monobasic potassium phosphate and mix well to dissolve. Bring to volume with deionized water, and verify 4.0<pH<5.0. Store refrigerated in glass. Stable 1 month.

g. Diethyl ether (High purity grade or equivalent)

h. Toluene (HPLC grade or equivalent)

i. Ether:Toluene (1:1 v:v):
Combine 50 mL HPLC grade toluene with 50 mL diethyl ether. Mix well. Store in glass at room temperature. Stable 1 month.

j. Chloroform (GC^2 grade or equivalent)

k. Methanol (Optima, GC^2 grade or equivalent)

l. Chloroform:Methanol ($CHCl_3$:MeOH) (4:1 v:v):
Combine 40 mL chloroform with 10 mL GC^2 methanol. Mix well. Store in brown glass at room temperature. Stable 1 month.

m. Ethanol (Pharmaceutical grade or equivalent)

n. Water (Deionized)

o. Ethanol 80% (v/v aqueous):
Measure 80 mL pharmaceutical grade ethanol into a 100-mL graduated cylinder. Bring to volume with deionized water and mix well. Store in glass at room temperature. Stable for 6 months.

p. Vortex mixer

q. Centrifuge

r. Rotator

s. Evaporator with nitrogen

t. Routine laboratory supplies, including disposable pipettes, wooden sticks, test tube racks, graduated cylinders, etc.

u. Gas Chromatograph / Mass Spectrometer equipped with a 30 m x 0.25 mm x 0.25 µm Rtx-5MS (or equivalent) column

v. Methylene chloride (Optima grade or equivalent)

w. 10 cc glass centrifuge tubes (with conical bottom)

6 Standards and Controls

a. Methylphenylhydantoin (MPH) Standard:
Purchased from Sigma-Aldrich or another approved vendor. Storage and stability determined by manufacturer.

b. Methylphenylhydantoin Stock Standard (1 mg/mL):
Add 10.0 mg of methylphenylhydantoin to a 10-mL volumetric flask. Dilute to the mark with methanol and mix well. Store refrigerated in glass. Stable for at least 2 years.

c. Methylphenylhydantoin Working Internal Standard (30 µg/mL):
Dilute 0.75 mL of the MPH Stock Standard to 25 mL with deionized water. Store refrigerated in glass. Stable for at least 2 years.

d. Octanoic Acid Standard:
Purchased from Sigma-Aldrich or another approved vendor. Storage and stability determined by manufacturer.

e. Octanoic Acid Working Internal Standard (1mg/mL):
Add 10.0 mg of octanoic acid to a 10-mL volumetric flask. Dilute to the mark with methanol and mix well. Store refrigerated in glass. Stable for at least 2 years.

f. Negative Control:
Purchased from Cliniqa or an equivalent supplier, or prepared in-house from an appropriate blank specimen. Store refrigerated or obtain fresh. Stability determined by manufacturer. A Negative Control will be extracted and analyzed with every assay. When possible, the Negative Control will be matrix matched.

g. Barbiturate Mix-5:
A mixture of five barbiturates at 250 µg/mL in methanol. Purchased from Cerilliant or another approved supplier. Contains amobarbital, butalbital, pentobarbital, phenobarbital, and secobarbital. This mixture may also be prepared from individual analyte stock solutions if necessary. Storage and stability determined by manufacturer.

h Positive Control Solution:
In addition to the Barbiturate Mix-5, the following target analytes are obtained from an approved vendor in liquid (1 mg/mL) or solid form. Storage and stability determined by the manufacturer. Solid analytes are dissolved in methanol or another appropriate solvent to prepare 1 mg/mL stock solutions. Analyte stock solutions (1 mg/mL) are added to a 25 mL volumetric flask which is brought to the mark with methanol as described in Table 2 below:

Table 2: Positive Control Solution and Control Preparation

Analyte(s)	Stock Conc. (mg/mL)	Spike Aliquot (µL)	Solution Volume (mL)	Solution Conc. (µg/mL)	Control Spike Aliquot (µL)	Matrix Volume (mL)	Control Conc. (ng/mL)
Barbiturate Mix-5	0.25	250	25	2.5	100	0.5	500
Carbamazepine	1	63	25	2.52	100	0.5	504
Carisoprodol	1	63	25	2.52	100	0.5	504
Ibuprofen	1	300	25	12	100	0.5	2400
Meprobamate	1	63	25	2.52	100	0.5	504
Phenytoin	1	63	25	2.52	100	0.5	504

The Positive Control Solution is stored refrigerated in glass or plastic. Stable for at least two years. (Note: Other drugs or metabolites may be added to this mixture as dictated by case needs with sufficient validation and/or analysis of concurrent controls.)

i. Positive Control:
100 µL of the Positive Control Solution is added to 0.50 mL of the Negative Control Blood or Urine on the day of analysis. Optional: 25 µL of a 1 mg/mL acetaminophen standard can be added directly to the Positive Control as well (yields a 50 µg/mL concentration).

A Positive Control will be extracted and analyzed with every assay. When possible, the Positive Control will be matrix matched. Additionally, the use of the MPH internal standard serves as a qualitative positive control for the individual specimen. Other positive controls preparations may be used as is appropriate.

j. Acetaminophen Standard (1 mg/mL):
Purchased as a 1 mg/mL solution in methanol from Cerilliant or another approved supplier. Storage and stability determined by the manufacturer.

k. Valproic Acid Standard (1 mg/mL):
Purchased as a 1 mg/mL solution in methanol from Cerilliant or another approved supplier. Storage and stability determined by the manufacturer.

l. Valproic Acid Working Solution (100 µg/mL)
Dilute 500 µL of the Valproic Acid Standard (1 mg/mL) to 5 mL in methanol. Storage and stability determined by the manufacturer.

m. Volatiles Positive Control (20 µg/mL):
100 µL of the Valproic Acid Working Solution (100 µg/mL) is added to 0.50 mL of the Negative Control.

A Volatiles Positive Control will be extracted and analyzed with every volatiles assay. When possible, the Volatiles Positive Control will be matrix matched. Additionally, the use of the Octanoic Acid internal standard serves as a qualitative positive control for the individual specimen.

7 Sampling

Not applicable.

8 Procedure

Appendix 1 contains an abbreviated version of this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

a. To properly labeled 16 x 125 mm screw-top tubes add 0.5 mL of biological fluid, 0.5 g of a prepared food homogenate, 0.5 mL of a prepared beverage dilution, or 1 g of prepared tissue homogenate (1:1 in deionized water). Also prepare Negative and Positive Controls for each matric being analyzed. Prepare a Volatiles Positive Control if performing volatiles analysis. Bring up to a volume of 1 mL with deionized water.

b. Add 25 µL of MPH Internal Standard Solution to biological fluid specimens, food homogenates and beverage dilutions.[2] For tissue specimens add 0.5 mL of MPH Internal

[2]Other internal standards may be substituted or added at relevant concentrations as appropriate,

Standard Solution. This results in MPH specimen concentrations of 1.5 µg/mL and 10 µg/g, respectively. For volatiles analysis, add 25 µL of Octanoic Acid Working Internal Standard Solution (1 mg/mL) to biological fluid specimens, food homogenates and beverage dilutions. (If volatiles analysis *only* is to be performed, only the Octanoic IS needs to be added.)

c. Add 1 mL of 5% KH_2PO_4 buffer solution to each specimen. Check pH to ensure pH is between 4 and 6.

d. Add 5 mL of ether:toluene (1:1) to each tube and extract for 20 minutes on a rotator. Centrifuge 5 minutes. Use a wooden stick to break up any emulsions that develop.

e. Transfer organic (top) layer to a 16 x 100 mm culture tube.

f. Evaporate the ether:toluene to dryness under a gentle stream of nitrogen at 50ºC. If analysis for volatile acidic drugs (e.g., ethchlorvynol or valproic acid) is desired, pause this evaporation at about 0.5 mL and analyze 1-2 µL by GC-MS / electron impact ionization (positive ion mode) with the instrumental conditions given below.

g. Transfer dried residue to a 10 cc glass centrifuge tube with two successive 1 mL washes of $CHCl_3$:MeOH (4:1). Evaporate this solvent to a dry residue under a gentle stream of nitrogen at 50ºC.

h. Reconstitute the dried residue in 2 mL hexane plus 200 µL 80% ethanol. Vortex for 30 seconds and centrifuge for 5 minutes.

i. Discard the hexane (top) layer, transfer the ethanol layer to a fresh 12 x 75 tube, and evaporate the ethanol layer to a dry residue under a gentle stream of nitrogen at 50ºC.

j. Reconstitute the residues with 25-50 µL of $CHCl_3$:MeOH (4:1) and analyze 1-2 µL by GC-MS/chemical ionization (positive ion mode) and/or GC-MS/electron impact ionization (operating conditions specified in Section 9) after confirming instrument is calibrated and in proper working condition.

k. Some acidic drugs are known to carryover from run to run. To clean out the GC system, analyze a methylene chloride blank at the beginning of every sequence and after the Positive Control.

l. Evaluate the data

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with sufficient validation and/or analysis of concurrent controls.

m. Forensic examiner for the case: review electronic data files for acid/neutral drug screens and record that review by writing "file reviewed", "peaks checked", or similar language on the total ion chromatogram along with their initials.

9 Instrumental Conditions

Appendix 2 contains an abbreviated version of the instrumental conditions in this procedure. This form may be used at the bench by the examiner or chemist performing the procedure. Instrumental conditions may be modified to account for particular analytes. Any modifications will be recorded in case notes.

9.1 Gas Chromatograph Parameters (Agilent)

Oven Parameters		Inlet and Carrier Parameters		Column Parameters	
temperature 1	45ºC	inlet temperature	220ºC	type	DB-5MS
hold 1	1 min	injection mode	split	length	30 m
ramp 1	25ºC/min	carrier gas	ultrapure helium	internal diameter	0.25 mm
temperature 2	150ºC	carrier mode	constant flow	film thickness	0.25 µm
hold 2	2 min	carrier flow	1.2 mL/min		
ramp 2 (volatiles)	15ºC/min (30ºC/min)	split flow	12 mL/min		
temperature 3	280ºC	split ratio	10:1		
hold 3	14 min				
total run time (volatiles)	29.87 min (25.53 min)				

9.2 Mass Spectrometer Parameters - Agilent (EI)

ionization mode	electron impact (+)	Source/quad temperature	230/150ºC
scan mode	full scan	transfer line temperature	280ºC
scan range (volatiles)	35 – 500 AMU (35 – 200 AMU)	solvent delay (nominal) (volatiles)	3 min (3.5 min)

10 Decision Criteria

10.1 Batch Decision Criteria

No analytes of interest will be detected in the Negative Control. For this purpose, analytes of interest are defined as those analytes that will be reported for this batch.
All analytes should be detected in the Positive Control.

10.2 Unknown Sample Decision Criteria

10.2.1 Chromatography

The peak of interest will show good chromatographic fidelity, with reasonable peak shape, width, and resolution. In order to be determined acceptable, a chromatographic peak in an unknown sample will compare favorably to a chromatographic peak of the same analyte in a known sample analyzed on the same system in the same or subsequent analytical runs. Additionally, the following two criteria should be met.

10.2.1.1 Retention Time

The retention time of the peak will be within ±2% of the retention time (relative or absolute, as appropriate) obtained from injection of a reference standard, an extracted Positive Control, or an appropriate deuterated analog.

10.2.1.2 Signal-to-Noise

To justify the existence of a peak, its baseline signal to peak-to-peak noise ratio will exceed 3. Further, the baseline signal for the peak of interest will be at least 10 fold greater than that for any observed peak at similar retention time in a Negative Control or solvent blank injected just prior to the sample.

10.2.2 Mass Spectrometry

The mass spectrum of the analyte of interest will match that of a reference standard or an extracted Positive Control. See the Guidelines for Comparison of Mass Spectra standard operating procedure (Tox 104) for further guidance.

10.3 Planned Action on QC Failure

Refer to Quality Control for Toxicology Examinations (TOX101) for guidance on action steps in the event of a quality control failure.

10.4 Reporting Cut-offs for College of American Pathologists (CAP) T Series and FTC Series:

See Quality Control for Toxicology Examinations (TOX101) for guidance on estimating the amount of an analyte in a specimen. When analyzing CAP T-Series or FTC specimens, if all decision criteria for an analyte of interest are met, but the concentration of butalbital, carbamazepine, carisoprodol, meprobamate, phenobarbital, phenytoin, and/or secobarbital is estimated to be below 1 µg/mL (or 5 µg/mL for acetaminophen) in two independent analyses, the

analyte will not be reported. Note: the second analysis may be a repeat of this procedure or via another validated procedure. A Positive Control at the Cut-off Level is recommended for the second analysis.

11 Calculations

Not applicable.

12 Measurement Uncertainty

Not applicable.

13 Limitations

a. Limit of Detection (LOD): Detection limits for common acidic and neutral analytes are listed in Table 2 below. Note: LODs were not evaluated below 100 ng/mL, so true LODs for this method may be lower than what is listed in the table.

Table 3:

Analyte	Blood LOD (µg/mL)	Urine LOD (µg/mL)
Acetaminophen	25	1
Amobarbital	0.1	0.1
Brompheniramine	0.5	>0.5
Bupropion	0.1	0.25
Butalbital	2.5	0.5
Carbamazepine	0.1	0.1
Carisoprodol	0.5	0.1
Citalopram	0.25	0.5
Clozapine	>0.5	0.25
Cyclobenzaprine	0.1	0.1
Diphenhydramine	0.1	0.1
Ibuprofen	5	1
Ketamine	0.1	0.1
Lamotrigine	2.5	1
Lidocaine	0.25	0.25
Meprobamate	0.5	0.25
Methadone	0.25	0.1
Mirtazapine	0.1	0.1
Naproxen	50	2.5
Pentobarbital	0.1	0.1

Phenobarbital	0.1	0.1
Phenytoin	0.25	0.1
Propoxyphene	0.1	0.1
Secobarbital	0.1	0.1
Theophylline	2.5	0.1

b. Interferences: None known. Grossly decomposed or putrefied samples, as well as samples that have been embalmed, may affect detection limits.

c. Note: This method is not appropriate for screening for salicylic acid.

14 Safety

Take standard precautions for the handling of chemicals and biological materials. Refer to the *FBI Laboratory Safety Manual* for guidance.

15 References

Baselt, R.C. and Cravey, R.H. *J Anal Tox*, 1977, *1*, 81-103.

Baselt, R.C., *Disposition of Toxic Drugs and Chemicals in Man*, 7th ed., Biomedical Publications: Foster City, California, 2004.

Foerster, E.H., Dempsey, J., and Garriott, J.C. *J Anal Tox*, 1979**,** *3*, 87-91.

Moffat, A.C., *Isolation and Identification of Drugs*, 2nd ed., Pharmaceutical Press: London, 1986.

Winek, C. *Drug and Chemical Blood-Level Data*, 1994.

Chemistry Unit Validation of Analytical Procedures (CUQA 11); FBI Laboratory Chemistry Unit Quality Assurance and Operations Manual.

Quantitation of Acidic Drugs (Tox 424); FBI Laboratory Chemistry Unit – Toxicology SOP Manual.

Quality Control for Toxicology Examinations (TOX101); FBI Laboratory Chemistry Unit – Toxicology SOP Manual.

Guidelines for Comparison of Mass Spectra (Tox 104); FBI Laboratory Chemistry Unit – Toxicology SOP Manual.

FBI Laboratory Chemistry Unit – Instrument Operation and Support SOP Manual.

FBI Laboratory Safety Manual.

Rev. #	Issue Date	History
7	04/01/19	Updated Table 2 for clarity and to include 'as-prepared' analyte concentrations. Section 9: added line clarifying that instrument conditions may be modified to target particular analytes, and that the modifications will be recorded in case notes.
8	11/15/19	Updated punctuation in Section 1. Added octanoic internal standard preparation and volatiles positive control preparation to Section 6. Updated positive control concentration in Table 2. Added control preparation step to Section 8.b. Added 10.4 CAP reporting cut-offs. Updated bench sheets.

Approval

Redacted - Signatures on File

Acting Toxicology Technical Leader: Date: 11/14/2019

Chemistry Unit Chief: Date: 11/14/2019

Appendix 1: Abbreviated version of the Acid/Neutral Procedure for bench use.

Redacted - Form on File

Appendix 2: Abbreviated version of the Acid/Neutral Instrumental Conditions for bench use.

Redacted - Form on File